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# ISCHEMIC PRECONDITIONING: A DEFENSE MECHANISM AGAINST THE REACTIVE OXYGEN SPECIES GENERATED AFTER HEPATIC ISCHEMIA REPERFUSION<sup>1</sup>

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**Background.** Preconditioning protects against both liver and lung damage after hepatic ischemia-reperfusion (I/R). Xanthine and xanthine oxidase (XOD) may contribute to the development of hepatic I/R.

**Objective.** To evaluate whether preconditioning could modulate the injurious effects of xanthine/XOD on the liver and lung after hepatic I/R.

**Methods.** Hepatic I/R or preconditioning previous to I/R was induced in rats. Xanthine and xanthine dehydrogenase/xanthine oxidase (XDH/XOD) in liver and plasma were measured. Hepatic injury and inflammatory response in the lung was evaluated.

**Results.** Preconditioning reduced xanthine accumulation and conversion of XDH to XOD in liver during sustained ischemia. This could reduce the generation of reactive oxygen species (ROS) from XOD, and therefore, attenuate hepatic I/R injury. Inhibition of XOD prevented postischemic ROS generation and hepatic injury. Administration of xanthine and XOD to preconditioned rats led to hepatic MDA and transaminase levels similar to those found after hepatic I/R. Preconditioning, resulting in low circulating levels of xanthine and XOD activity, reduced neutrophil accumulation, oxidative stress, and microvascular disorders seen in lung after hepatic I/R. Inhibition of XOD attenuated the inflammatory damage in lung after hepatic I/R. Administration of xanthine and XOD abolished the benefits of preconditioning on lung damage.

**Conclusions.** Preconditioning, by blocking the xanthine/XOD pathway for ROS generation, would confer protection against the liver and lung injuries induced by hepatic I/R.

Ischemia-reperfusion (I/R) is an unavoidable process in liver transplantation, and protection of liver against injuries

resulting from ischemia-reperfusion (I/R) remains one of the major nonimmunologic problems of liver transplantation (1, 2). One of the mechanisms thought to cause hepatic injury during I/R is the formation of reactive oxygen species (ROS). Potential sources of ROS are the activation of Kupffer cells and neutrophils (3), the oxidation of heme proteins under the acid conditions associated with ischemia (4), and xanthine/xanthine oxidase (XOD) (5). During ischemia, xanthine dehydrogenase (XDH) is converted to the oxygen radical-producing form, XOD. Concurrently, there is an accumulation of the substrate for XOD, xanthine. Upon reoxygenation, XOD reacts with molecular oxygen to produce ROS, which mediate subsequent injurious events (6). It is well known that during I/R processes, antioxidant systems such as glutathione (GSH) and superoxide dismutase (SOD) could be depleted or inactivated, respectively, leading to an increased vulnerability of liver to ROS damage (7, 8).

On the other hand, the injurious effects of ROS are not limited to the liver. There is evidence indicating that the release of xanthine and XOD from the liver into the bloodstream plays an essential role in the pathogenesis of systemic complications of hepatic I/R, including neutrophil infiltration and oxidant stress into the lung (5, 9, 10). Consequently, any mechanism with the capability to act on the liver xanthine/XOD system, either by reducing the conversion rate of XDH to XOD or the amount of substrates available to XOD could be considered useful to attenuate the injurious effects of ROS seen in liver and lung as a consequence of hepatic I/R.

An inducible potent endogenous mechanism against I/R injury has been termed ischemic preconditioning. This phenomenon, commonly studied in the heart by Murry et al. in 1986 (11), has been also demonstrated in other organs, such as brain (12), intestine (13), and liver (14). Despite intensive investigations, however, the mechanisms responsible for the protective effects of preconditioning remain unresolved. A potential mechanism responsible for the protective effect of preconditioning could be the induction of endogenous defense systems against ROS. In the heart and intestine, several studies have reported that ischemic preconditioning could attenuate the ROS increase in tissues subsequently exposed to prolonged I/R (15–18). These reduction in ROS levels have been explained by an increase in the antioxidants pool (SOD or GSH), which remove ROS (15–17), or by the effect of preconditioning on ROS-generating systems such as neutrophils (15) or xanthine/XOD (18). However, there is controversial data in the literature, because the results obtained by

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other authors in the heart seem to indicate that the effectiveness of ischemic preconditioning could not be explained by an increased myocardial resistance to ROS (19–21).

To our knowledge, the possibility that preconditioning could modulate the injurious effects of ROS after hepatic I/R has not been determined. Recently, we reported that ischemic preconditioning attenuated both the liver and lung injuries associated with hepatic I/R (22, 23). Our hypothesis suggests that this process would be effected by modulating those mechanisms involved in both local and systemic disorders, including xanthine and XOD. The deleterious effects of xanthine could not be exclusively limited to the ROS generation from XOD. Previous studies indicate that xanthine is also an inhibitor of adenosine receptors (24). It is known that the activation of adenosine receptors by ischemic preconditioning or by pharmacological treatments may induce the nitric oxide synthesis, thus reducing the hepatic I/R injury in warm and cold ischemia conditions (25–27).

The present study evaluates (1) whether ischemic preconditioning acting on the tandem xanthine/XOD, by decreasing the amount of xanthine and/or the conversion of XDH to XOD in liver, could protect against hepatic injury induced by I/R; (2) the role of antioxidant system, SOD and GSH, in the protection conferred by preconditioning on hepatic injury; and (3) whether ischemic preconditioning could modulate the levels of circulating xanthine and/or XOD, thus attenuating the lung injury induced by hepatic I/R. Understanding the mechanisms involved in ischemic preconditioning could help to provide new surgical and pharmacological strategies to protect against hepatic I/R injury.

## MATERIAL AND METHODS

### *Surgical Procedure*

The study was performed on male Wistar rats weighing between 250 and 300 g. Animals were anesthetized with urethane (10 mg/kg, i.p.) and placed in a supine position on a heating pad to maintain body temperature at 36–37°C. To induce hepatic ischemia, laparotomy was performed and the blood supply to the right lobe of the liver was interrupted by placement of a bulldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp (14). This study was performed in concordance with the European Union regulations (Directive 86/609 EEC) for animal experiments.

### *Experimental Design*

To evaluate the effect of preconditioning on ROS generating and antioxidant systems in liver after hepatic ischemia, animals were distributed into the following groups:

1. Control (C) (n=6): animals subjected to anesthesia and laparotomy.
2. Ischemia (I) (n=6): animals subjected to 90 min of right-lobe hepatic ischemia.
3. Preconditioning + ischemia (PC+I) (n=6): same as group 2 but with preconditioning induced by 10 min of ischemia followed by 10 min of reperfusion before ischemia.

After the sustained ischemia, liver samples were obtained, immediately frozen, and maintained at –80°C until analytical determinations of SOD, GSH, xanthine, and XDH/XOD activity.

To evaluate the effect of preconditioning on the release of xanthine and XOD from the liver into the bloodstream after restoration of blood flow, animals subjected to the same procedures as in groups 2 and 3 were subjected to 15 min of reperfusion after 90 min of ischemia. After 15 min of hepatic reperfusion, blood samples were

obtained from the cava vein and processed to determine xanthine and XDH/XOD activity.

To study whether the effect of preconditioning on ROS generating and/or antioxidant systems could modulate the postischemic ROS generation and consequently the hepatic I/R injury associated with this process, animals were distributed into the following groups:

4. Ischemia-reperfusion (I/R) (n=6): animals subjected to 90 min of right-lobe hepatic ischemia (as in group 2) followed by 90 min of reperfusion.
5. PC+I/R (n=6): same as group 4 but with previous preconditioning induced by 10 min of ischemia and 10 min of reperfusion before sustained ischemia.
6. I/R+allopurinol (n=6): same as group 4 but with prior administration of an inhibitor of xanthine oxidase, allopurinol (100 mg/kg, portal vein) 15 min before reperfusion (28).
7. PC+I/R+xanthine+XOD (n=6): same as group 5, but with a continuous intravenous infusion of xanthine (5 mM; 0.033 ml/min) and xanthine oxidase (0.2 U/L; 0.033 ml/min) (portal vein) for 30 min, 5 min before the start of reperfusion (29). The two drugs were maintained in separate syringes and they reached a common catheter just before entering the bloodstream.
8. PC+I/R+allopurinol+xanthine+XOD (n=6): same as group 5 but with a continuous intravenous infusion of xanthine (5 mM; 0.033 ml/min) and xanthine oxidase (0.2 U/L; 0.033 ml/min) for 30 min, 5 min before the start of reperfusion and with prior administration of allopurinol at dose of 100 mg/kg 15 min before reperfusion.
9. I/R+glutathione ester (I/R+GSH) (n=6): same as group 4 but with administration of GSH ester (5 mmol/kg, portal vein) 5 min before reperfusion (30).

At the end of 90 min of reperfusion, blood, liver, and lung samples were obtained. Blood samples were collected to determine plasma aminotransferases (aspartate aminotransferase [AST], alanine aminotransferase [ALT]). Liver samples were processed to determine lipoperoxides (MDA), SOD, and GSH. Lung samples were processed to determine MDA and myeloperoxidase (MPO) activity. Vascular permeability analysis in lung and histological analysis in liver and lung were performed.

The reperfusion time to evaluate the release of xanthine and XOD from the liver into the bloodstream after restoration of blood flow was as described by Weinbroum et al. (9). The dose and pretreatment times of GSH ester administration has been reported to be effective in evaluate the effects of GSH depletion on hepatic I/R injury. GSH itself is not suitable because GSH is not taken up by cells in its intact form, because it is degraded into its constituent amino acids before entering the cells. The GSH ester is readily permeable to cells. Once inside the cell cytosol the ester is hydrolyzed by an esterase to yield GSH. The ester is most effective in increasing hepatic GSH levels (30).

Control experiments were performed with the vehicles containing the different drugs. Allopurinol, xanthine, XOD, and GSH ester were supplied by Sigma Chemical Co. (St. Louis, MO).

### *Biochemical Determinations*

**Xanthine measurement.** For the analysis of xanthine concentration in liver, tissues were homogenized in 3.6% perchloric acid. After homogenization, tissues were allowed to extract for 30 min at 0.5°C. For the analysis of xanthine in plasma, 500  $\mu$ l of blood was placed in 1.0 ml of 3.6% perchloric acid at 5°C for 30 min. After centrifugation, the supernatants (corresponding to liver and plasma samples) were neutralized with a potassium carbonate-potassium hydroxide solution to pH 6.5–7.0. The assay mixture consisted of  $\text{Na}_2\text{CO}_3$  (0.05 M, pH 10.2), EDTA ( $10^{-4}$  M); nitroblue tetrazolium ( $1.5 \times 10^{-4}$  M), and XOD (100 U/L). The reduction of nitroblue tetrazolium to formazan was recorded at 560 nm (31).

**XDH and XOD activity.** Liver tissues were homogenized in 100 mM Tris, containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenate was then spun down at 15,000 g at 4°C and the pellet discarded. The supernatant

was chromatographed on Sephadex G-25 80 column in the same buffer at 4°C (32). To analyze XDH and XOD in plasma samples, a total of 500  $\mu$ l of plasma supernatant was passed through a Sephadex G-25 80 column. The resultant eluates (corresponding to liver and plasma samples) were used for measurement of XDH and XOD activity. Activities were measured spectrophotometrically on the basis of uric acid formation at 292 nm in the presence or absence of 0.60 nM NAD<sup>+</sup>, respectively. Xanthine (60 mM) was used as substrate. The kinetics of the reaction was recorded for 10 min at 20°C.

**Glutathione measurement.** For the analysis of GSH, liver samples were homogenized in 1.1% KCl. Proteins were precipitated with 1 N perchloric acid. After centrifugation, samples were neutralized with 10% K<sub>2</sub>CO<sub>3</sub> (18). The amount of GSH was measured using glutathione transferase and 1-chloro-2,4-dinitrobenzene (33). Fifty microliters of the previous treated sample was mixed with 225  $\mu$ l of 0.1 M potassium phosphate buffer, pH 7.0, and 10  $\mu$ l of 10 mM 1-chloro-2,4-dinitrobenzene in ethanol. The reaction was started with 5  $\mu$ l of glutathione transferase solution (12 U/L) and monitored at 340–400 nm reaching the end-point 5 min after enzyme addition.

**Superoxide dismutase assay.** For SOD determination, liver samples were homogenized in 100 mM Tris (hydroxymethyl) aminomethane (Tris)-HCl buffer with 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (pH 8.1). SOD activity was assayed by determining the ability of the enzyme to inhibit the superoxide anion-mediated reduction of nitroblue tetrazolium (25  $\mu$ M) to formazan, according to the method of Sun et al. (34). The later was determined spectrophotometrically at 560 nm. The superoxide anion required for this reaction is generated by xanthine (0.1 mM) and xanthine oxidase (200 U/L).

**Lipid peroxidation assay.** Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by ROS (35). Lipid peroxidation in liver and lung samples was determined by the thiobarbiturate (TBA) reaction measuring the formation of malondialdehyde (MDA). For this purpose, 2 ml of trichloroacetic acid (20%)

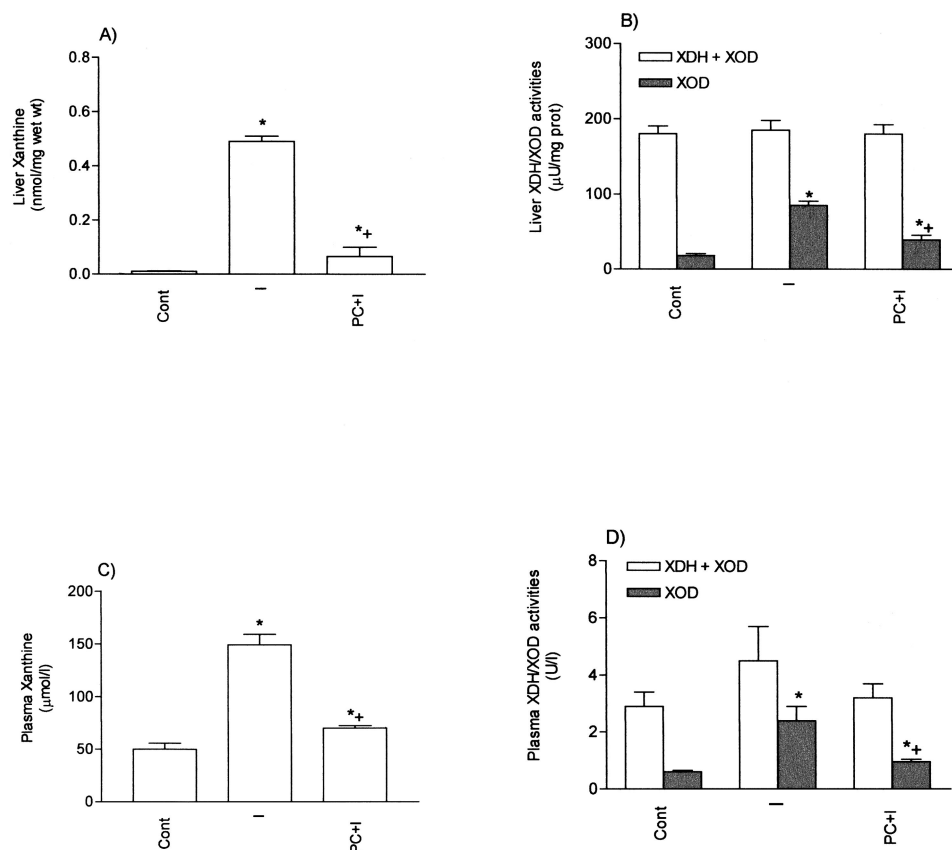
was added to 2 ml of homogenate. After mixing and centrifuging, 1 ml of TBA-water solution (0.67%) was added to the supernatant and boiled for 60 min. After cooling, optical density at 530 nm was assayed (23, 36).

**Hepatic injury.** Evaluation of hepatic injury was performed by enzymatic determinations of AST and ALT plasma levels using a commercial kit from Boehringer Mannheim (Munich, Germany).

**Myeloperoxidase assay.** Myeloperoxidase (MPO) has been used as a marker of pulmonary neutrophil infiltration and activation (37). MPO activity was measured photometrically employing 3,3',5,5'-tetramethylbenzidine as a substrate (38). Lung samples were macerated with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer pH 6. Homogenates were then disrupted by sonication for 30 sec and subsequently snap frozen in dry ice and thawed three times before a final 30 sec sonication. Samples were incubated at 60°C for 2 hr and then spun down at 4000 $\times$ g for 12 min. Supernatants were collected for MPO assay. Enzyme activity was assessed photometrically. The assay mixture consisted in 20  $\mu$ l supernatant, 10  $\mu$ l tetramethylbenzidine (final concentration 1.6 mM) dissolved in DMSO, and 70  $\mu$ l H<sub>2</sub>O<sub>2</sub> (final concentration 3.0 mM) diluted in 80 mM phosphate buffer pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of one absorbance unit per minute.

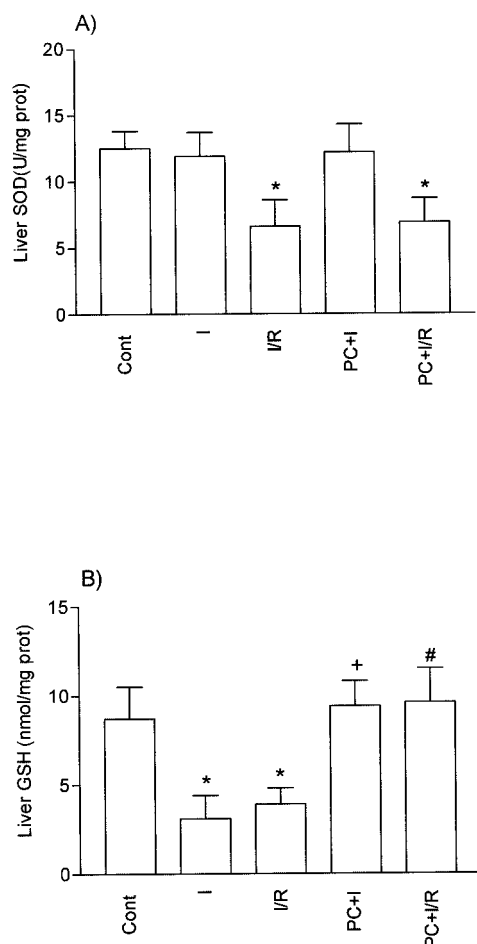
**Vascular permeability analysis.** Vascular permeability in lung was estimated using the Evans Blue method. Animals received 20 mg/kg of Evans Blue by cava vein injection 15 min before sacrifice. Lung samples were added to 10 volumes of deionized formamide and incubated at room temperature for 24 hr and the Evans Blue extracted from tissue was quantitated by spectrophotometric analysis and compared to results with standards of known concentrations (22).

**Protein measurement.** Total protein concentration in liver homogenates was determined using a commercial kit from Bio-Rad (Munich, Germany).



**FIGURE 1.** Hepatic xanthine levels (A) and XDH/XOD activity (B) after ischemia and plasma xanthine (C) and XDH/XOD activity (D) immediately after hepatic reperfusion. Cont, Control; I, 90 min of ischemia; PC+I, I with previous preconditioning induced by 10 min of ischemia and 10 min of reperfusion before sustained ischemia. \* $P < 0.05$  vs. Cont; + $P < 0.05$  vs. I.





**FIGURE 2.** Hepatic SOD activity (A) and GSH (B) levels in the following experimental groups: Cont, Control; I, 90 min of ischemia; I/R, 90 min of ischemia followed by 90 min of reperfusion; PC+I, I with previous preconditioning induced by 10 min of ischemia and 10 min of reperfusion before sustained ischemia; PC+I/R, I/R with previous preconditioning induced by 10 min of ischemia and 10 min of reperfusion before sustained ischemia. \* $P < 0.05$  vs. Cont; + $P < 0.05$  vs. I; # $P < 0.05$  vs. I/R.

#### Histological Study

Liver and lung samples were obtained, fixed in 10% neutral buffered formalin, paraplast-embedded, cut into 5- $\mu$ m sections, and stained with hematoxylin-eosin according to standard procedures.

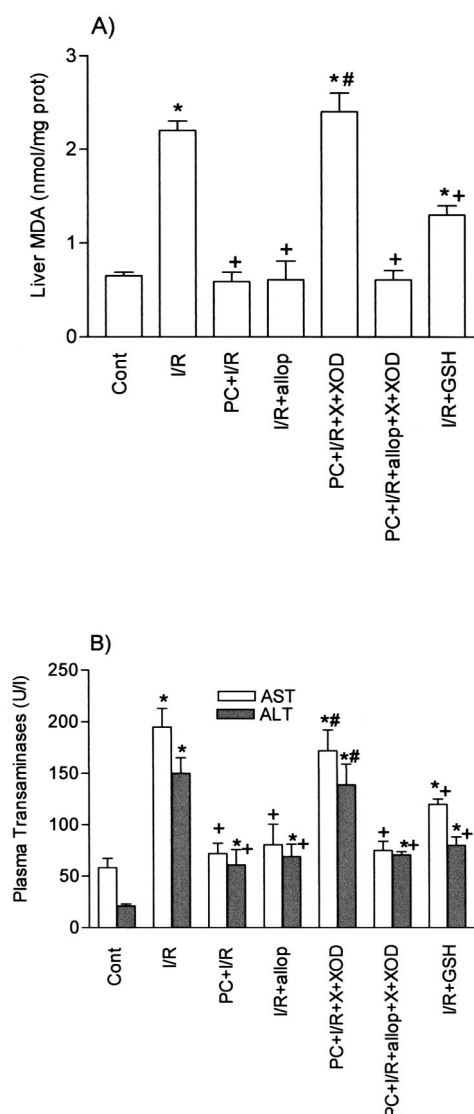
#### Statistics

Data are expressed as mean  $\pm$  SEM. Mean of different groups were compared using a one-way analysis of variance. Student's *t* test was performed for evaluation of significant differences between groups. Significance was determined at the 5% level ( $P < 0.05$ ).

### RESULTS

#### ROS Generating Systems in Liver Tissue and Plasma

As shown in Figure 1, a significant increase in xanthine levels in liver (Fig. 1A) was observed after hepatic ischemia (I). With regard to XDH and XOD activity in liver (Fig. 1B), no change in total XDH+XOD activity was observed. However, increased conversion of XDH to XOD occurred as a consequence of ischemia, because this proportion increased



**FIGURE 3.** Hepatic MDA levels (A) and hepatic injury (B) after reperfusion in the following experimental groups: Cont, Control; I/R, 90 min of ischemia followed by 90 min of reperfusion; PC+I/R, I/R with previous preconditioning; I/R+allop, I/R+allopurinol; PC+I/R+X+XOD, PC+I/R+xanthine and xanthine oxidase; PC+I/R+allop+X+XOD; I/R+GSH, I/R+glutathione ester. \* $P < 0.05$  vs. Cont; + $P < 0.05$  vs. I/R; \*\* $P < 0.05$  vs. PC+I/R.

to 40%. Preconditioning previous to ischemia (PC+I) attenuated both the increase in xanthine levels and the conversion of XDH to XOD, because XOD represented only 20% of the total enzymatic activity. As expected, an increase in xanthine and XOD activity in plasma (Fig. 1, C and D, respectively) has been shown at a few minutes after hepatic reperfusion. When preconditioning was carried out, the increases in both xanthine and XOD activity were attenuated.

#### ROS Antioxidant Systems in Liver

SOD and GSH represent well known enzymatic and non-enzymatic antioxidant mechanisms (39, 40). We have evaluated whether preconditioning, by acting through these antioxidant mechanisms, could confer resistance against the

injury induced by the ROS generated after hepatic I/R. As shown in Figure 2A, SOD activity levels found after ischemia (I) were of the same order as in the control group but a significant decrease in SOD levels was observed after hepatic reperfusion (I/R). Preconditioning did not modify the decrease in SOD activity, suggesting that the differences in ROS observed between nonpreconditioned and preconditioned groups could not be explained by differences in SOD activity. With regard to GSH (Fig. 2B), ischemia leads to a reduction in hepatic GSH levels with respect to control group which was not modified after hepatic reperfusion. However, when ischemia was preceded by preconditioning, GSH levels were similar to those of the control group. We proceeded to evaluate whether a preconditioning period could modify by itself the GSH contents before the sustained ischemia but no differences in GSH levels with respect to control group were found (data not shown); this suggests that the differences in the GSH content found at the end of ischemia in animals subjected or not to previous preconditioning, may result either in differences in the synthesis or consumption of GSH during the ischemic process.

#### *Role of Xanthine/XOD and GSH on Postischemic ROS and Hepatic Injury After Hepatic I/R*

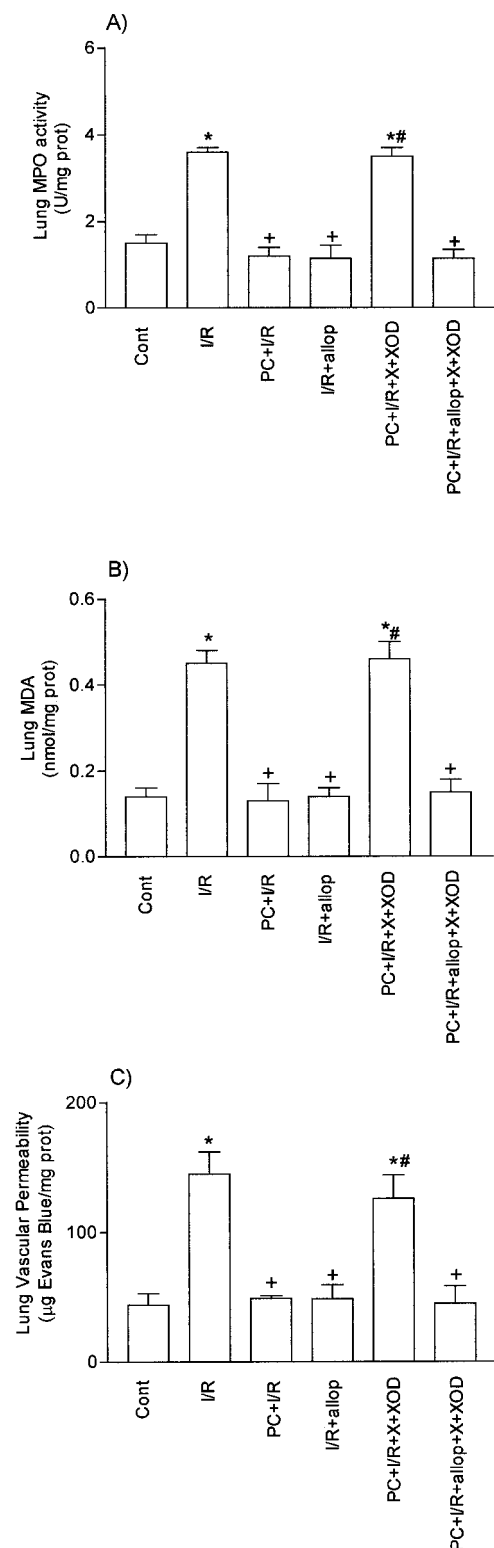
Hepatic I/R resulted in the generation of ROS, as shown by MDA levels significantly higher than those found in the control group (Fig. 3A). These increases in postischemic MDA levels were prevented when preconditioning was carried out (PC+I/R). We investigated whether preconditioning by blocking the xanthine/XOD system during prolonged ischemia could confer protection against hepatic injury. The inhibition of XOD with allopurinol prevented the ROS generation (I/R+allop). The administration of xanthine and XOD to preconditioning group (PC+I/R+X+XOD) resulted in MDA levels similar to those found after hepatic reperfusion, whereas the inhibition of XOD in this group (PC+I/R+allop+X+XOD) prevented the effects, resulting in levels like those found by preconditioning (see Fig. 3A). An identical pattern was observed for hepatic injury (AST, ALT), as illustrated in Figure 3B. We evaluated whether the maintenance of GSH content after hepatic ischemia induced by preconditioning could contribute to attenuate the injuring effects of ROS in I/R processes. The administration of GSH ester to I/R (I/R+GSH) resulted in MDA and transaminase levels significantly lower than those obtained after hepatic reperfusion.

#### *Role of Circulating Xanthine/XOD on Lung Injury After Hepatic I/R*

As shown in Figure 4, either preconditioning or the inhibition of XOD with allopurinol attenuated the increases in MPO (Fig. 4A), MDA (Fig. 4B), and vascular permeability (Fig. 4C) observed after hepatic I/R. The administration of xanthine and XOD (PC+I/R+X+XOD) abolished the benefits of preconditioning, leading to MPO, MDA, and vascular permeability values similar to those found after hepatic I/R. In contrast, the administration of allopurinol (PC+I/R+allop+X+XOD) abolished the adverse effects of xanthine/XOD.

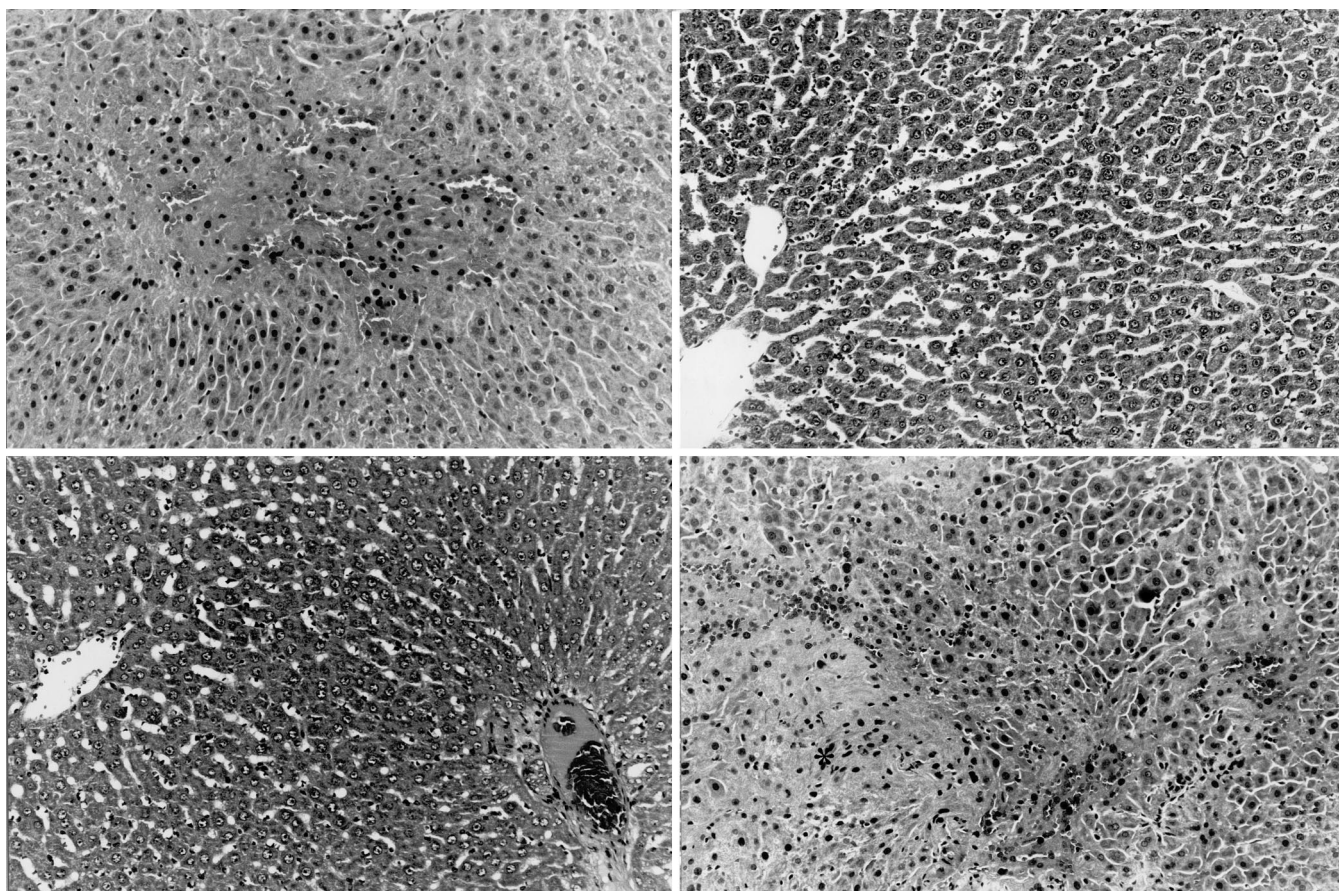
#### *Histological Study of the Liver and Lung after Hepatic I/R*

Histological changes in both liver and lung after hepatic I/R were in keeping with the biochemical study (Figs. 5 and



**FIGURE 4.** MPO (A), MDA (B), and vascular permeability (C) in lung after hepatic I/R. Cont, Control; I/R, 90 min of ischemia followed by 90 min of reperfusion; PC+I/R, 90 min of ischemia followed by 90 min of reperfusion with previous preconditioning induced by 10 min of ischemia and 10 min of reperfusion before sustained ischemia; I/R+allop, I/R+allopurinol; PC+I/R+X+XOD, PC+I/R+xanthine and xanthine oxidase; PC+I/R+allop+X+XOD. \* $P < 0.05$  vs. Cont; + $P < 0.05$  vs. I/R; # $P < 0.05$  vs. PC+I/R.





**FIGURE 5.** Histological lesions in liver after hepatic I/R. (A) I/R: focal areas of hepatocyte necrosis; (B) PC+I/R: no hepatic lesions; (C) I/R+allop: no hepatic lesions; (D) PC+I/R+X+XOD: focal areas of hepatocyte necrosis and disruption of hepatic architecture (\*) (hematoxylin and eosin stain; magnification  $\times 255$ ).

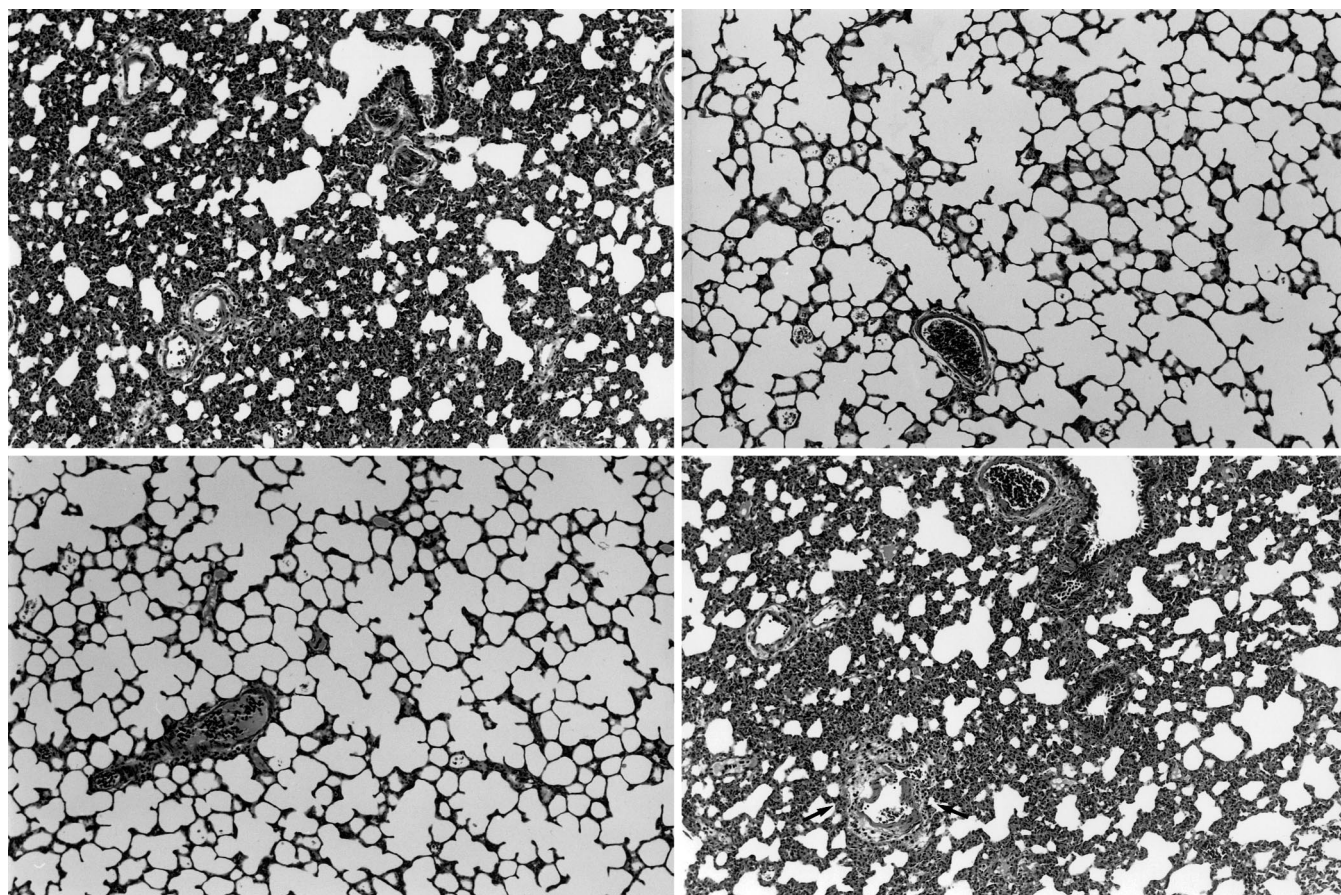
6). The histological study of the liver in the I/R (Fig. 5A) and PC+I/R+X+XOD (Fig. 5D) groups revealed multiple and extensive areas of hepatocyte necrosis randomly distributed through the hepatic parenchyma. In contrast, no hepatic lesions or minimal lesions were observed in PC+I/R (Fig. 5B) and I/R+allop (Fig. 5C). In lung, the I/R (Fig. 6A) and PC+I/R+X+XOD (Fig. 6D) showed a moderate thickening of the alveolar walls with neutrophil infiltration. By contrast, no pulmonary lesions or minimal lesions were seen in PC+I/R (Fig. 6B) and I/R+allop (Fig. 6C).

#### DISCUSSION

The destructive effects of I/R arise from the acute generation of ROS subsequent to reoxygenation, which inflict direct tissue damage and initiate a cascade of deleterious cellular responses leading to inflammation, cell death, and organ failure (41, 42). It has been suggested that one of the main sources of ROS during the I/R process lies in the activity of XOD (5). According to the model proposed by Granger et al. (43), XDH is converted to XOD when liver tissue is subjected to ischemia (Fig. 1). The accumulation of xanthine found after hepatic ischemia could thus be deleterious in hepatic I/R through the enhanced generation of ROS by the newly formed XOD. Inhibition of XOD with allopurinol (I/R+allop) attenuates the production of ROS, reducing also the hepatic

I/R injury. The results of the present study indicate that preconditioning limited the accumulation of xanthine and reduced the conversion of XDH to XOD during prolonged ischemia. However, the administration of xanthine and XOD to the preconditioned group (PC+I/R+X+XOD) abolished the benefits of preconditioning on oxidative stress and hepatic injury. MDA, transaminase levels, and histological results similar to those found after hepatic I/R were found (Figs. 3 and 5). Taking into account these results, xanthine should abolish the protection conferred by preconditioning due to the higher levels of ROS generated by XOD. Thus, we inhibited XOD in the preconditioned group pretreated with X+XOD to determine if this brings back the preconditioning protection. As shown in Figure 3, the injurious effects of xanthine+XOD were prevented with previous inhibition of XOD (PC+I/R+allop+X+XOD), resulting in MDA and transaminase values similar to those obtained by preconditioning. This data indicate that preconditioning could reduce the postischemic ROS production by blocking xanthine/XOD, conferring thus protection against hepatic I/R injury. Conversely, the deleterious effects of xanthine could also be the result of preventing nitric oxide production by its antagonism of the adenosine receptor. This possibility cannot be discounted, because it has been reported that xanthine is also an inhibitor of adenosine receptors (24), and the protective role of adenosine receptor activation on hepatic I/R injury is





**FIGURE 6.** Histological lesions in lung after hepatic I/R. (A) I/R: Moderate thickening of the alveolar walls with neutrophil infiltration; (B) PC+I/R: no apparent pulmonary lesions; (C) I/R+allop: no apparent pulmonary lesions; (D) PC+I/R+X+XOD: moderate thickening of the alveolar walls with neutrophil infiltration and perivascular neutrophil extravasation (arrows) (H & E stain; magnification  $\times 128$ ).

well known (25–27). In our previous work, in the same experimental model used in the present study, we demonstrated that preconditioning induces the activation of adenosine A2 receptors, which by eliciting an increase in nitric oxide generation before sustained ischemia, protect against the hepatic injury observed at 90 min of hepatic reperfusion (25). This endogenous production of nitric oxide could confer protection by modulating mechanisms involved in hepatic I/R injury, including sustained hepatic vasoconstriction, which compromises long-term recovery from hepatic I/R injury (44). Nitric oxide is also involved in the protection conferred by preconditioning on I/R injury associated with liver transplantation (45). In addition, adenosine receptor agonists increase nitric oxide production, and this confers a protective PC-like effect (14, 25, 27). This effect has been reported by other investigators in the liver (26), who go one step further and demonstrate that adenosine receptor antagonists oppose this effect.

Because GSH is involved in both chemical and enzymatic cellular antioxidant defense mechanisms, a decrease in this metabolite during prolonged ischemia (Fig. 2) is consistent with a decline in the ability of cells to attenuate damage by ROS. As shown in Figure 3, administration of GSH resulted in ROS and transaminase levels significantly lower than those obtained after hepatic I/R. Conversely, the maintenance

of GSH induced by preconditioning after prolonged ischemia could also contribute to the protection against hepatic injury.

Experimental evidence indicates that hepatic I/R is associated with the release of XOD and xanthine from the liver into the circulation. Then, the resulting XOD-derived oxidants may act as mediators of the inflammatory response in the lung (5, 9, 10). Neutrophils have been demonstrated to accumulate in the lung after hepatic I/R. Activated neutrophils can produce oxidants and result in acute lung injury, including increased alveolar-capillary membrane permeability (9, 10, 23). The inhibition of XOD with allopurinol prevented the neutrophil accumulation, oxidative stress, and microvascular disorders seen in lung after hepatic I/R (Figs. 4 and 6). Values of MPO, MDA, and microvascular permeability of the same order as found in control groups were obtained. All of these data suggest that XOD may play a pivotal role in remote tissue damage. This contention is consistent with the observation that circulating xanthine and XOD is increased in patients with adult respiratory distress syndrome (ARDS) associated with human liver transplantation and that ARDS patients have circulating neutrophils that are activated and sequestered in the lungs (9, 10). Preconditioning reduced levels of circulating xanthine and XOD (Fig. 1). This could confer protection against the pulmonary



damage after hepatic I/R. As shown in Figure 4, the administration of xanthine and XOD to the preconditioned group (PC+I/R+X+XOD) abolished the beneficial effects of preconditioning, leading to neutrophil accumulation, values of oxidative stress, and microvascular disorders similar to those found after hepatic I/R. Histological changes in lung were in keeping with the biochemical study (Fig. 6). PC+I/R+X+XOD group (Fig. 6D) showed a moderate thickening of the alveolar walls with neutrophil infiltration, whereas no lesions were observed in lung tissue of the PC+I/R group (Fig. 6B). The deleterious effects of xanthine may be due to ROS generation from XOD, because the previous inhibition of XOD with allopurinol (PC+I/R+allop+X+XOD) did not modify the benefits of preconditioning.

The results of the present study give support to the idea that preconditioning could protect against both the hepatic and the lung injury associated with hepatic I/R by modulating the mechanisms implicated in the local and systemic inflammatory response. Recently, we reported that preconditioning reduced the postischemic hepatic and plasma tumor necrosis factor (TNF) release from Kupffer cells (22). However, the reduction in TNF release was important in the protection conferred by preconditioning in lung injury, but this would not completely explain the protection conferred by preconditioning in liver (23). It is known that hepatic I/R injury is a result of the multiple interactions between different mechanisms (2, 44). The results of the present study seem to indicate that the effect of preconditioning on xanthine/XOD could also contribute to attenuate both the liver and lung injury after hepatic I/R.

In liver transplantation, the preservation-reperfusion injuries are still one of the most serious insults that affect postoperative graft viability and ultimate postsurgical results (1, 2). The amount of xanthine and XOD as well as the loss of GSH during organ preservation seem to be responsible for ROS generation and organ damage after liver transplantation (5, 8). To date, in clinical practice, addition of XOD inhibitors, such as allopurinol or antioxidants such as GSH in the preservation solution, is not enough to eliminate the deleterious effects of ROS generated later during reperfusion (5, 46). The present experimental study suggests that the benefits of organ preconditioning could be of clinical interest, because this surgical approach prevents the formation of factors responsible for ROS production. Of course, intensive investigations are necessary to evaluate if these results obtained in normothermic conditions at 90 min of ischemia and reperfusion periods could be extrapolated to long term I/R conditions as well as to the cold ischemia conditions associated with liver transplantation. If this were possible, preconditioning, by reducing xanthine accumulation and conversion of XDH to XOD as well as by suppressing the loss of glutathione during organ preservation, could prevent ROS production, thus protecting liver grafts from subsequent I/R injuries. In addition, preconditioning by modulating the levels of circulating xanthine and XOD could attenuate the systemic effects of hepatic I/R, such as neutrophil infiltration, oxidant stress, and microvascular disorders into the lung.

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## REFERENCES

1. Delva E, Camus Y, Nordlinger B, et al. Vascular occlusions for liver resections. Operative management and tolerance to hepatic ischemia; 142 cases. *Ann Surg* 1989; 209: 211.
2. Clavien PA, Harvey PRC, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992; 53: 957.
3. Jaesche H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; 260: G355.
4. Harrison SC, Blout ER. Reversible conformational changes of myoglobin and apomyoglobin. *J Biol Chem* 1965; 240: 299.
5. Pesonen EJ, Linder N, Raivio KO, et al. Circulating xanthine oxidase and neutrophil activation during human liver transplantation. *Gastroenterology* 1998; 114: 1009.
6. Stirpe F, Della Corte E. The regulation of rat liver xanthine oxidase. Conversion in vitro of the enzyme activity from dehydrogenase (type D) to oxidase (type O). *J Biol Chem* 1969; 244: 3855.
7. Franssen C, Defraigne JO, Detry O, Pincemail J, Deby C, Lamy M. Antioxidant defense and free radical production in a rabbit model of kidney ischemia-reperfusion. *Transplant Proc* 1995; 27: 2880.
8. Vreugdenhil PK, Belzer FO, Southard JH. Effect of cold store on tissue and cellular glutathione. *Cryobiology* 1991; 28: 143.
9. Weinbroum A, Nielsen VG, Tan S, et al. Liver ischemia-reperfusion increases pulmonary permeability in rat: role of circulating xanthine oxidase. *Am J Physiol* 1995; 268: 988.
10. Weiland JE, Davis WB, Holter JF, et al. Lung neutrophils in adult respiratory distress syndrome: clinical and pathophysiologic significance. *Am Rev Respir Dis* 1986; 133: 218.
11. Murry C, Jennings R, Reimer K. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74: 1124.
12. Heurteaux C, Lauritzen I, Widmann C, Lazdunski M. Essential role of adenosine, adenosine A<sub>1</sub> receptors, and ATP-sensitive K<sup>+</sup> channels in cerebral ischemic preconditioning. *Pharmacology* 1995; 92: 4666.
13. Hotter G, Closa D, Prados M, et al. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun* 1996; 222: 27.
14. Peralta C, Hotter G, Closa D, et al. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: role of nitric oxide and adenosine. *Hepatology* 1997; 25: 934.
15. Hoshida S, Kuzuya T, Yamashita N, et al. Brief myocardial ischemia affects free radical generating and scavenging systems in dogs. *Heart Vessels* 1993; 8: 115.
16. Yamashita N, Nishida M, Hoshida S, et al. Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. *J Clin Invest* 1994; 94: 2193.
17. Das DK, Liu X, Jones RM, Prasad R. Preconditioning of heart by repeated stunning induces oxidative stress protection and attenuates myocardial reperfusion injury. *FASEB J* 1991; 5: A8.
18. Sola A, Hotter G, Prats N, et al. Modification of oxidative stress in response to intestinal preconditioning. *Transplantation* 2000; 69: 767.
19. Turrens JF, Thornton J, Barnard M, et al. Protection from reperfusion injury by preconditioning hearts does not involve increased antioxidant defense. *Am J Physiol* 1992; 262: H585.
20. Omar BA, Hanson AK, Bose SK, McCord JM. Ischemic preconditioning is not mediated by free radicals in the isolated rabbit heart. *Free Radic Biol Med* 1991; 11: 517.
21. Tang XL, Qiu Y, Turrens JF, et al. Late preconditioning against stunning is not mediated by increased antioxidant defenses in conscious pigs. *Am J Physiol* 1997; 273: H1651.
22. Peralta C, Prats N, Xaus C, et al. Protective effect of liver ischemic preconditioning on liver and lung injury induced by hepatic ischemia-reperfusion in the rat. *Hepatology* 1999; 30: 1484.
23. Peralta C, Fernández L, Panés J, et al. Preconditioning protects against systemic disorders associated with hepatic ischemia-reperfusion through blockade of tumor necrosis factor-induced P-selectin up-regulation in the rat. *Hepatology* 2001; 33: 100.
24. Schwabe U, Ukena D, Lohse MJ. Xanthine derivatives as antagonists at A1 and A2 adenosine receptors. *Naunyn Schmiedeberg Arch Pharmacol* 1985; 330: 212.
25. Peralta C, Hotter G, Closa D, et al. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemic preconditioning is mediated by activation of adenosine A2 receptors. *Hepatology* 1999; 29: 126.

26. Nakayama H, Yamamoto Y, Kume M, et al. Pharmacologic stimulation of adenosine A<sub>2</sub> receptor supplants ischemic preconditioning in providing ischemic tolerance in rats liver. *Surgery* 1999; 126: 945.
27. Arai M, Thurman RG, Lemasters JJ. Contribution of adenosine A<sub>2</sub> receptors and cyclic adenosine monophosphate to protective ischemic preconditioning of sinusoidal endothelial cells against storage/reperfusion injury in rat livers. *Hepatology* 2000; 32: 297.
28. Green CJ, Healing G, Simpkin S, et al. Allopurinol inhibits lipid peroxidation in warm ischaemia and reperfused rabbit kidneys. *Free Radic Res Commun* 1989; 6: 329.
29. Hotter G, Closa D, Prats N, et al. Free radical enhancement promotes leukocyte recruitment through a PAF and LTB<sub>4</sub> dependent mechanism. *Free Rad Biol Med* 1997; 22: 947.
30. Grattagliano I, Vendemiale G, Lauterburg BH. Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. *J Surg Res* 1999; 86: 2.
31. Folch E, Salas A, Panés J, et al. Role of P-selectin and ICAM-1 in pancreatitis-induced lung inflammation in rats: significance of oxidative stress. *Ann Surg* 1999; 230: 792.
32. Corte ED, Stirpe F. The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. *Biochem J* 1972; 126: 739.
33. Brigelius R, Muckel C, Akerboom TPM, Sies H. Identification and quantification of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* 1983; 32: 2529.
34. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. *Clin Chem* 1988; 34: 497.
35. Baykal A, Kaynaroglu V, Demirpençe E, et al. Experimental study of the effect of adrenaline tolerance on intestinal ischaemia-reperfusion. *Br J Surg* 1998; 85: 947.
36. Peralta C, Rull R, Rimola A, et al. Endogenous nitric oxide and exogenous nitric oxide supplementation in hepatic ischemia-reperfusion injury in the rat. *Transplantation* 2001; 71: 529.
37. Cutrin JC, Boveris A, Cíngaro B, et al. In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment. *Hepatology* 2000; 31: 622.
38. Trush MA, Egnor PA, Kensler TW. Myeloperoxidase as a biomarker of skin irritation and inflammation. *Fed Chem Toxic* 1994; 32: 143.
39. Portolés MT, Catalá M, Antón A, Pagani R. Hepatic response to the oxidative stress induced by *E. coli* endotoxin: glutathione as an index of the acute phase during the endotoxic shock. *Mol Cell Biochem* 1996; 159: 115.
40. Rees R, Smith DJ, Adamson B, et al. Oxidant stress: the role of the glutathione redox cycle in skin preconditioning. *J Surg Res* 1995; 58: 395.
41. Connor HD, Gao W, Nukina S, et al. Evidence that free radicals are involved in graft failure following liver transplantation in the rat-electron paramagnetic resonance spin trapping study. *Transplantation* 1992; 54: 199.
42. Sumimoto K, Matsura T, Oku JI, et al. Protective effect of UW solution on postischemic injury in rat liver: suppression of reduction in hepatic antioxidants during reperfusion. *Transplantation* 1996; 27: 1391.
43. Granger DNJ, Rutili G, McCord JM. Role of superoxide radicals in intestinal ischemia. *Gastroenterology* 1981; 81: 22.
44. Serracino-Inglott F, Aviv NA, Mathie RT. Hepatic ischemia-reperfusion injury. *Am J Surg* 2001; 181: 160.
45. Yin DP, Sankary HN, Chong ASF, et al. Protective effect of ischemic preconditioning on liver preservation-reperfusion injury in rats. *Transplantation* 1998; 66: 152.
46. Changani KK, Fuller BJ, Bell JD, et al. Improved preservation solutions for organ storage. *Transplantation* 1999; 68: 345.

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